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PROTEIN ROTATION AND CHROMOPHORE ORIENTATION IN RECONSTITUTED BACTERIORHODOPSIN VESICLES

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Summary

Bacteriorhodopsin has been reconstituted into lipid vesicles with dipalmitoyl and dimyristoyl phosphatidylcholine. Circular dichroism (CD) measurements show that the proteins are in a monomeric state above the main lipid phase transition temperature (T_c), 41 and 23°C for dipalmitoyl and dimyristoyl phosphatidylcholine, respectively. Below T_c, the CD spectrum is the same as that found for the purple membrane. The latter result implies that the orientation of the chromophore at these temperatures is most likely the same as in the purple membrane (70° ± 5° from the normal to the membrane plane).

Transient dichroism measurements show that below T_c the proteins are immobile, while above this temperature protein rotation around an axis normal to the plane of the membrane is occurring. In addition, from the data the angle of the chromophore for the rotating proteins with respect to the rotational diffusion axis can be calculated. This angle is found to be 30° ± 3° and 29° ± 4° in dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine, respectively. This is considerably smaller than the value of 70° ± 5° for the natural biomembrane. A reversible reorientation of the chromophore above and below the respective main T_c transition temperature could explain the change of angle observed provided that all the molecules rotate above T_c.

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Introduction

Bacteriorhodopsin in the purple membrane of *Halobacterium halobium* is present in a hexagonal lattice [1—3]. Flash photoselection studies have shown that the protein is immobile within that structure [4—6] and the dipole moment of the chromophore, all-*trans* retinal in the case of the light-adapted state, forms an angle of about 70° ± 5° with the normal of the membrane [6—8]. If bacteriorhodopsin is reconstituted in dipalmitoyl or dimyristoyl phosphatidylcholine vesicles, it has been shown that whilst above the phase transition the lattice disaggregates and the proteins rotate as monomers, provided the lipid: protein molar ratio is high enough [9], below the phase transition temperature of the respective lipid the protein crystallizes into patches sometimes having the same hexagonal lattice as observed in the purple membrane.

In the present paper, we compare and contrast our results with those previously reported for the angle of the transition moment for rotating bacteriorhodopsin molecules in the reconstituted vesicles [7].

Materials and Methods

Growth of *H. halobium* R1 (kindly supplied by Dr. R. Henderson) and purple membrane preparation were performed according to the procedure given by Oesterhelt and Stoeckenius [10].

The method of membrane reconstitution is similar to that described by Hesketh et al. [12] for the Ca^{2+}-ATPase from sarcoplasmic reticulum. Two substitution steps were performed. In the first, purple membrane (10 mg protein/ml) was suspended together with dimyristoyl or dipalmitoyl phosphatidylcholine (2 mg/mg protein) in a 150 mM KCl, 20 mM potassium acetate buffer, pH 6, and sodium cholate (2 mg/mg protein). After incubation for 30 min at 27 and 45°C for dimyristoyl and dipalmitoyl phosphatidylcholine, respectively, the samples were layered over a continuous sucrose density gradient (15—60%, w/v) and centrifuged at 53 000 × *g* for 4 h. The temperature for the centrifugation was 27°C for dimyristoyl phosphatidylcholine and 38°C for dipalmitoyl phosphatidylcholine. After centrifugation, the purple band was collected and washed with buffer. For the second substitution step the procedure was repeated, only the amount of cholate was varied from 1 to 7 mg/mg protein. This enabled recombinants having a lipid : protein molar ratio in the range 9 : 1 up to 180 : 1 to be prepared. For flash photolysis studies, the samples were resuspended in 150 mM KCl, 20 mM acetate (pH 5) buffer, containing 66% glycerol (v/v). Protein was assayed according to the method of Lowry et al. [17] using bovine serum albumin as a standard. Lipid was assayed by quantitative gas-liquid chromatography [18]. The instrument used was a Pye Unicam Series 204 gas chromatograph equipped with a column of 15% (w/w) poly(ethylene glycol adipate) on Gas-Chrom Z. The high concentration of glycerol was chosen in order to have no interference from smaller vesicles which might tumble in aqueous suspensions in the time scale of milliseconds.

The flash photolysis apparatus is described elsewhere [13]. Briefly, bacteriorhodopsin is excited by a plane-polarized laser flash. Transient absorbance changes for light parallel ($A_\parallel$) and perpendicular ($A_\perp$) with respect to the polar-

isation of the exciting flash are calculated from the simultaneously measured transmittance changes, $T_{\parallel}$ and $T_{\perp}$. The data are analysed by calculating the anisotropy parameter $r(t)$, given by $r(t) = A_{\parallel}(t) - A_{\perp}(t)/A_{\parallel}(t) + 2A_{\perp}(t)$. The samples for flash photolysis normally had an absorbance of 0.7—1.5 at the measuring wavelength (570 nm).

In order to test our apparatus for possible artefacts, we prepared bacteriorhodopsin monomers [11] which are rotating in the nanosecond time scale. Purple membranes (approx. 10 mg/ml) were sonicated in the dark for 10 min in a 150 mM KCl, 20 mM potassium acetate buffer, pH 6.0, containing 10% (v/v) Triton X-100 at about 40°C using a bath sonicator (Dawe Instruments Type 6441) in order to solubilize the protein. After centrifugation at 100 000 × g for 30 min to remove any residual purple membrane fragments, the supernatant was collected and used without further treatment.

CD measurements were performed using a Cary 61 spectropolarimeter equipped with a variable temperature cell.

Results

Excitation of bacteriorhodopsin by a short laser pulse results in transient absorbance changes in the sample [14,15]. At 570 nm these are given by negative changes due to ground-state depletion.

In order to check our apparatus for possible artefacts, Triton X-100-solubilized bacteriorhodopsin was flashed. As shown in Fig. 1a, the difference

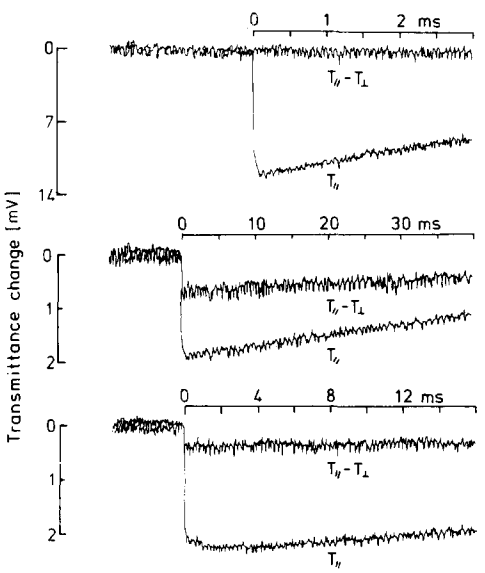

Fig. 1. Transient transmittance changes, T, from bacteriorhodopsin (a) solubilised in Triton X-100 in buffer without 66% glycerol, (b) and (c) reconstituted with dimyristoyl phosphatidylcholine at a molar ratio of 54:1 (mol lipid:mol protein) in 150 mM KCl, 20 mM acetate buffer, pH 5, containing 66% glycerol (v/v) at 15 and 30°C, respectively. Flash (20 ns, 530 nm) at time t =0. The initial voltage was 120 mV in the case of a and 60 mV for b and c. The measuring wavelength was (575 ± 6) nm and the sample had an absorbance of about 1.0 at this wavelength. The larger signal in all cases is the parallel polarized component, $T_{\parallel}$, the smaller signal (corresponding to the baseline in a) the difference $T_{\parallel}$—$T_{\perp}$ between the parallel and perpendicular polarized components of the measuring beam.

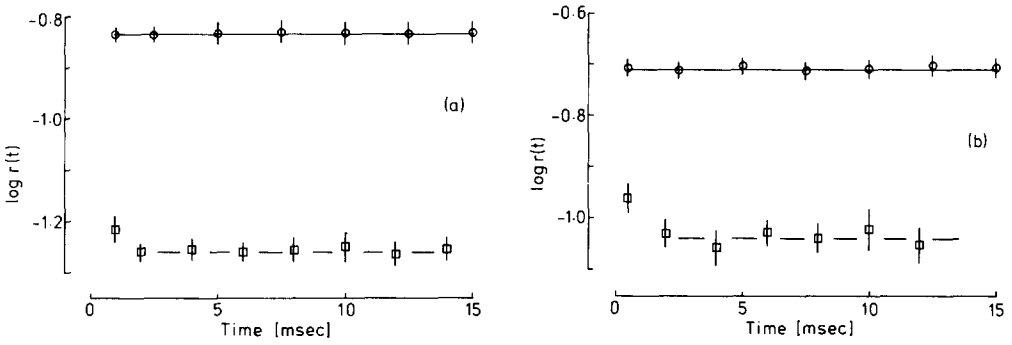

Fig. 2. Time dependence of the anisotropy parameter, $r(t)$, calculated as mean value ±S.D. from three subsequent measured signals: (a) dimyristoyl phosphatidylcholine:bacteriorhodopsin molar ratio (54:1) at 15°C (○———○) and 30°C (□———□); (b) dipalmitoyl phosphatidylcholine:bacteriorhodopsin molar ratio (71:1) at 20°C (○———○) and 46°C (□———□).

signal, $\Delta T = T_{\parallel} - T_{\perp}$, is always zero in our time scale (milliseconds). This is to be expected because the isotropic rotational motion of the solubilised bacteriorhodopsin [11] is expected to have a rotational diffusion time in the nanosecond time domain. Hence, both measuring beams are always equal on the micro- to millisecond time scale.

If bacteriorhodopsin is reconstituted with dimyristoyl phosphatidylcholine or dipalmitoyl phosphatidylcholine, a flash-induced dichroism is always detected. Typical results for a dimyristoyl phosphatidylcholine:bacteriorhodopsin (molar ratio 54 : 1) flashed at 15 and 30°C are shown in Fig. 1b and c, respectively. After calculating the anisotropy parameter, $r(t)$, its logarithm is plotted vs. time and shown in Fig. 2a. The evaluation for a dimyristoyl phosphatidylcholine:bacteriorhodopsin (71 : 1) recombinant is presented in Fig. 2b. It should be noted that each datum point in Fig. 2 is the mean value (±S.D.) from three anisotropy parameters calculated from three signals taken at each respective temperature.

The main phase transition temperature, T_c, for dimyristoyl phosphatidylcholine is 23°C, for dipalmitoyl phosphatidylcholine 41°C. If measurements are performed below these temperatures (at 15°C for dimyristoyl phosphatidylcholine vesicles, 20°C for dipalmitoyl phosphatidylcholine vesicles) the anisotropy parameter does not decay noticeably over 40 ms. This demonstrates that the protein is immobilized in the time scale of the experiment and is in agreement with the finding by Heyn et al. [7]. Above T_c, i.e., at 30 and 46°C, respectively, a very fast decay for the anisotropy is detected which does not decay to zero but rather stays constant over at least 20 ms (r_∞). All results are reversible with temperature.

Following an approach given by Cherry et al. [16], we may describe the time dependence of r for an intrinsic protein rotating around an axis normal to the plane of the membrane as follows:

$$r(t) = A\exp(-D_\perp t) + A_2\exp(-4D_\perp t) + A_3 \quad (1)$$

where $A = (\frac{6}{5})\,(\sin^2\gamma\,\cos^2\gamma)$; $A_2 = (\frac{3}{10})\,(\sin^4\gamma)$; $A_3 = (\frac{1}{10})\,(3\cos^2\gamma - 1)^2$; D is the diffusion coefficient for rotation around an axis normal to the membrane;

and γ the angle between transition moment and normal to the membrane.

If all molecules in the sample rotate, the time-independent anisotropy, r_∞, coincides with A_3. Hence, the angle γ can be calculated [7] according to:

$$\gamma = \arccos((1 \pm 2m^{1/2})/3)^{1/2} \quad (2)$$

where m is the ratio between time-independent anisotropy r_∞ and flash-induced anisotropy r_0.

A complication arises if two populations of molecules are present: one which is rotating, the other which is immobile (i.e., if it is present within aggregates which are too large to show rotation on the time scale of the experiment). In this case, the time dependence for the anisotropy is given by:

$$r(t) = r^{\mathrm{mob}}(t)\,(1-\alpha) + r^{\mathrm{imm}}\alpha \quad (3)$$

where $r^{\mathrm{mob}}(t)$ is given by Eqn. 1, $r_\infty = A_3(1-\alpha) + r^{\mathrm{imm}}\alpha$ is the measured time-independent anisotropy, $r(t=0) = r_0$, and α is the fraction of immobile molecules.

If one assumes that the angle γ is the same for molecules rotating and immobile, it is easy to show that the fraction, α, of immobile molecules is given by:

$$\alpha = (r_\infty - mr_0)/(r_0(1-m)) \quad (4)$$

where m is given by rearranging Eqn. 2:

$$m = (\tfrac{1}{4})\,(3\cos^2\gamma - 1)^2 \quad (5)$$

Consequently, we have two possible ways of interpreting our data. We can assume (a) that the transition moment in bacteriorhodopsin is the same for rotating and immobile molecules, hence calculating the proportion of immobile proteins, or (b) that all molecules are rotating above the phase transition of the respective lipid, and hence calculating the angle.

Our CD measurements show that, below the transition temperature of the lipid, a negative maximum at 600 nm occurs. This is indicative of excitonic coupling, such as occurs in the protein hexagonal lattice of purple membrane [9]. Upon heating the sample above the lipid transition temperature, the

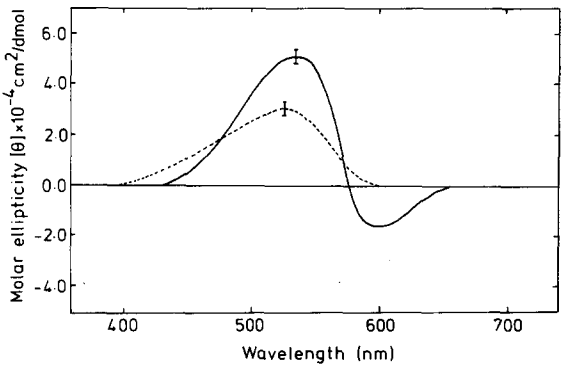

Fig. 3. CD spectrum for a dipalmitoyl phosphatidylcholine:bacteriorhodopsin (54:1 molar ratio) at different temperatures, 25°C (———) and 45°C (- - - - -)

negative maximum is lost, due to a breakdown of the protein lattice (see Fig. 3). These results are in agreement with previous findings by Cherry et al. [9] and show that the sample does not contain appreciable amounts of protein aggregates or areas of native purple membrane.

Calculation of immobile fraction

Assuming the chromophore in the rotating bacteriorhodopsin molecules forms the same angle γ as measured in the purple membrane ($\gamma = 70 \pm 5°$ [6—8]), we are able, according to Eqn. 4, to calculate the fraction of immobile proteins. We have used the method used by Heyn et al. [7] to obtain the flash-induced and time-independent anisotropy parameters, r_0 and r_∞, respectively. The latter is read directly from the graph above the respective transition temperature and the first is taken from the measurement below this temperature. Below the phase transition, the proteins are immobile and r does not decay from its initial value r_0 [7].

For the dimyristoyl phosphatidylcholine:bacteriorhodopsin (54:1) sample, we find $r^{imm}/r_0 = 0.29$, and for the dipalmitoyl phosphatidylcholine:bacteriorhodopsin (71:1) sample a value of 0.40. Consequently, we would have to postulate that 29% in the dimyristoyl phosphatidylcholine sample and 40% in the dipalmitoyl phosphatidylcholine sample are immobile (rotational correlation time slower than 20 ms) whereas in the case of dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine samples, respectively, 71 and 60% of the bacteriorhodopsin molecules are rotating with a relaxation time faster than 20 μs (as estimated from measurements taken at higher time resolution, unpublished results).

Calculation of angle γ

If we assume that molecules do rotate in our recombinants, the determination of the angle, γ, according to Eqn. 2 is straightforward. The value we obtain are $30° \pm 3°$ for dimyristoyl phosphatidylcholine and $29° \pm 4°$ for dipalmitoyl phosphatidylcholine if we take the positive sign in front of the square root in Eqn. 2. If we take the negative sign we obtain an imaginary solution which is rejected.

Discussion

Above the lipid transition temperature, freeze-fracture electron microscopy of our samples shows only a random distribution of small particles with no large aggregates. The lack of excitonic coupling above the respective main transition temperatures in the CD measurements suggests the absence of purple membrane fragments. Furthermore, for decreasing lipid:bacteriorhodopsin molar ratios, the value r_∞/r_0 increases (indicative of bacteriorhodopsin clusters), whereas at increasing molar ratios r_∞/r_0 remains unchanged.

It thus seems unlikely that we should interpret our data in terms of aggregates and that an interpretation based upon a change of angle is more likely. The two values we obtain for dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine recombinants on this basis are the same within experimental error. This value of about 29° is different from the result given recently

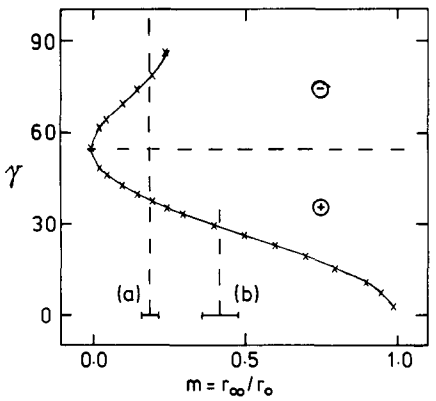

Fig. 4. Dependence of the angle, γ (between the axis of rotational diffusion and the transition moment of the chromophore all-*trans* retinal), on $m = r_\infty/r_0$ according to Eqn. 2. In region ⊕, the values of γ are calculated taking the + sign, for the ⊖ region taking the — sign in front of the square root in $\gamma = \arccos [(1 \pm 2m)^{1/2}/3]^{1/2}$: (a) result by Heyn et al. [7], $m = 0.19 \pm 0.03$; (b) our result, $m = 0.42 \pm 0.06$ (mean value ±S.D. from dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine recombinant experiments).

by Heyn et al. [7], who gave a value of 78° for this angle, as calculated from the measured ratio $m = r_\infty/r_0$ of 0.19. From Fig. 4, which illustrates the dependence of γ on m as stated in Eqn. 2, up to $m = 0.25$ two values of γ are possible, 38° and 78° corresponding to the ratio $m = 0.19$. The first value was rejected by Heyn et al. [7] as it did not agree with the angle observed with the purple membrane, when bacteriorhodopsin is in its hexagonal lattice, and the second value, $\gamma = 78°$, was assumed to be appropriate. Our measured value of m does not allow two possible values of γ (see fig. 4). Hence, it may be possible that the value of $\gamma = 38°$, rejected by Heyn et al. [7], is really the appropriate one for the reconstituted systems. The small difference then occurring between our value for the angle and theirs may arise from the different methods used for preparing the recombinants (dialysis method in Ref. 7, centrifugation method in our case).

CD spectroscopy [9] shows that the bacteriorhodopsin molecules are rotating as monomers above the lipid phase transition provided the lipid:protein molar ratio is high enough. The bacteriorhodopsin molecules once taken away from their hexagonal lattice, where they are mainly held together by protein-protein interactions, may then undergo a conformeric change which leads to a reversible reorientation of the chromophore.

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